Screening of antifeedant activity in brain extracts led to the identification of sulfakinin as a satiety promoter in the German cockroach

Are arthropod sulfakinins homologous to vertebrate gastrins–cholecystokinins?

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The feeding cycle of the adult female cockroach *Blattella germanica* parallels vitellogenesis. The study of the mechanisms that regulate this cycle led us to look for food-intake inhibitors in brain extracts. The antifeedant activity of brain extracts was tested *in vivo* by injecting the extract and measuring the carotenoids contained in the gut from carrot ingested after the treatment. By HPLC fractionation and tracking the biological activity with the carrot test, we isolated the sulfakinin EQFDDY(SO_3H)GHMRFamide (Pea-SK). A synthetic version of the peptide inhibited food intake when injected at doses of 1 μg (50% inhibition) and 10 μg (60% inhibition). The sulfate group was required for food-intake inhibition. These biological and structural features are similar to those of the gastrin–cholecystokinin (gastrin–CCK) family of vertebrate peptides. However, heterologous feeding assays (human CCK-8 tested on *B. germanica*, and Pea-SK tested on the goldfish *Carassius auratus*) were negative. In spite of this, alignment and cluster analysis of these and other structurally similar peptide families suggest that sulfakinins and gastrin–CCKs are homologous, and that mechanisms of feeding regulation involving these regulatory peptides may have been conserved during evolution between insects and vertebrates.

Keywords: *Blattella germanica*; food-intake control; German cockroach; sulfakinin.

How do insects regulate food intake and satiety? The answer holds an obvious academic interest, but it may also offer great potential for the design of new insecticides. Indeed, the induction of immediate satiety may prevent the attack of insects on our crops and stored food.

In vertebrates, a variety of mechanisms are involved in the regulation of food intake and energy balance, for example, physiological signals such as neuronal inputs related to circadian rhythms, metabolic signals reflecting the rate of use of various energy sources by brain and abdominal viscera, gastrointestinal signals elicited by gastric distension, and the release of peptides from the gastrointestinal tract in response to nutrient ingestion that act on the brain [1,2]. Most of these factors are peptides, which may be peripheral, central or circulatory. Satiety factors include: the short-term and peripheral peptides cholecystokinin (CCK), bombesin, enterostatin and glucagon-like peptide-1; the central peptides corticotropin-releasing factor and melanocortin; and the long-term circulatory proteinaceous factor leptin. Conversely, hunger factors are central and include neuropeptide Y, galanin and orexin [3].

The equivalent information has not been acquired for insects, although recent developments indicate that the endocrine regulation of physiological processes is at least as complex as in vertebrates. Therefore, thorough research in this field may unveil the factors involved in the presumably complex regulatory systems governing food intake in insects.

The adult females of anautogenous insect species, which require food as a prerequisite for oogenesis, are suitable experimental models for these studies. One such insect is the German cockroach, *Blattella germanica* [4], in which the feeding cycle of the adult female precedes oocyte development, with a peak of food consumption around the middle of the gonadotropic cycle [5]. This cyclic pattern suggests that feeding is finely regulated by factors influencing food intake. We thus studied these putative factors in brain extracts from adult females of *B. germanica* collected after the peak of food consumption.

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Abbreviations: CCK, cholecystokinin; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; SK, sulfakinin; Lem-SK-II, leucosulfakinin-II; Pea-SK, perisulfakinin.

Note: a web page is available at http://www.ibmb.csic.es

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MATERIALS AND METHODS

Insect rearing and tissue collection

Adult females of *B. germanica* were obtained from a colony fed on dog chow and water, and reared in the dark at 30 ± 1 °C and 60–70% relative humidity. Brains from 5- to

7-day-old adult virgin females were dissected free of optic lobes and adhering fat body, and homogenized in methanol/water/acetic acid (87 : 8 : 5, v/v/v). After centrifugation (8000 ***g*** for 10 min at 4 °C), the supernatant was collected and stored at −20 °C until use.

Feeding bioassay

One-day-old adult females of *B. germanica* were starved for 48 h, injected with the extract (or the standard compound) dissolved in Ringer saline, and immediately provided with carrot *ad libitum*. Controls received the same treatment but they were injected with Ringer saline alone. After 5 h in the presence of carrot, the whole gut was dissected out and extracted in methanol to quantify carotenoids following published methods [6]. Essentially, the gut was homogenized in 200 μL methanol and centrifuged (16 600 ***g*** for 5 min at 8 °C), and the methanol layer containing the crude pigment was recovered. The procedure was repeated (usually 3–6 times) until the pellet was white. Methanol fractions were pooled, and A_{450} was measured in a Labsystems Multiscan Plus spectrophotometer (Helsinki, Finland). Before standardizing the 5 h duration of the assay, we assessed that after 5 h of carrot feeding, carotenoids were not yet detected in the faeces. The total weight of carrot ingested was estimated by interpolation on a standard curve constructed using methanolic extracts of increasing amounts of lyophilized carrot. All results are expressed as mean ± SEM.

Preparation of crude extracts for preliminary biological tests

Under our rearing conditions, *B. germanica* shows a feeding peak on day 4 of the reproductive cycle [5]. Therefore, brains from 5- to 7-day-old adult females were analysed for a factor that inhibits food intake. The brains were dissected and extracted as described above, and loaded on a C_{18} solid-phase extraction column (Isolute, IST, Mid Glamorgan, UK) , usually in pools of 35. The column was eluted with various concentrations of acetonitrile in water. The fraction 17–40% acetonitrile, which retained the antifeedant activity, was used in the assays.

HPLC purification of the active factor

A total of 3000 brains from 5- to 7-day-old females were extracted and purified in four consecutive HPLC steps as summarized in the Fig. 1. Steps 1, 2 and 3 were carried out with a Merck–Hitachi (Darmstad) low-pressure system, L-6200A pump with a L-4200 UV-VIS detector. Step 4 was carried out with a Waters (Milford, MA, USA) low-pressure system, 626 pump with a 600S controller and 996 PDA detector. In the first three steps, fractions were separated every 2 min and tested with the carrot assay for food-intake inhibition. At least 10 replicates were carried out per assay. In the last step, carrot tests were performed on isolated peaks. The concentration of extract injected was 10, 25, 50 and 75 brain-equivalents per specimen, in the first, second, third and fourth steps, respectively. Once the peak responsible for the activity had been determined, the purification was repeated with a new extract of 2300 brains in order to identify the compound.

MS and sequencing

An aliquot of the fraction responsible for the biological activity was analysed using an Applied Biosystems Voyager DE-RP matrix-assisted laser desorption ionization time of

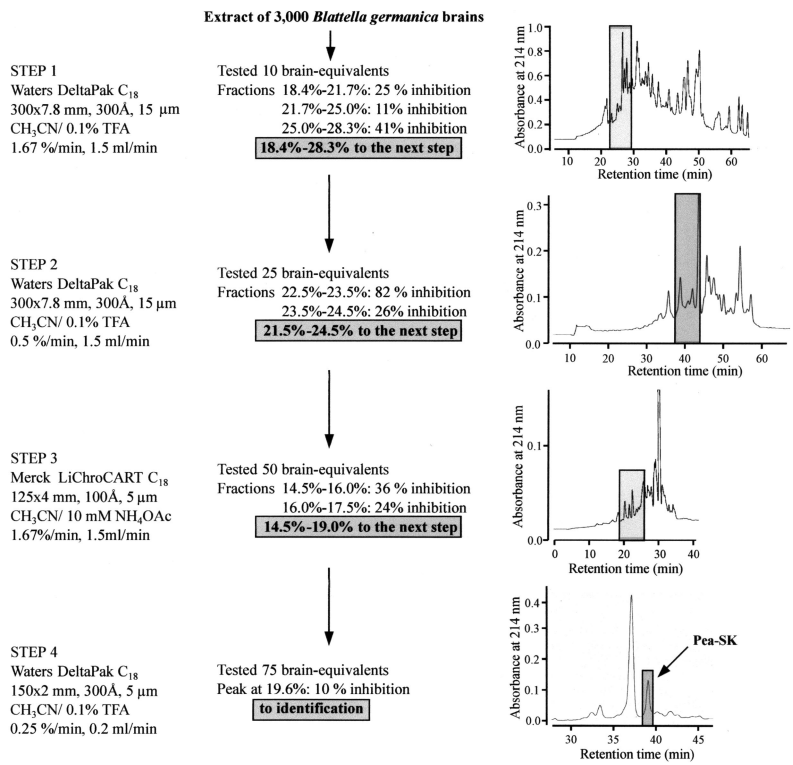

Fig. 1. Flow diagram summarizing the conditions for HPLC purification of the brain factor that inhibits food intake in *B. germanica*. Fractions with biological activity are highlighted. TFA, trifluoroacetic acid; NH_4OAc, ammonium acetate. Once the peak responsible for the activity had been determined, the process of purification was repeated with a new extract from 2300 brains to identify the compound as EQFDDY(SO_3H)GHMRFamide (Pea-SK).

flight (MALDI-TOF) mass spectrometer (Foster City, CA, USA). The sample was analysed in both the positive and negative modes. The amino-acid sequence of the purified factor was determined by Edman degradation using a Beckman LF-3000 sequencer (Palo Alto, CA, USA).

Synthetic peptides

The sulfakinin (SK) pQSDDY(SO_3H)GHMRFamide [leucosulfakinin-II (Lem-SK-II)] was synthesized on a solid polyethyleneglycol-polystyrene support (0.18 mmol·g^{-1}) functionalized with the xanthenyl amide linker [7] handle using Fmoc-based chemistry adapted for sulfopeptide synthesis [8]. The following side-chain protections for trifunctional residues were used: Asp(OtBu); His(Boc); Tyr(SO_3H). Arg, Met and Ser were unprotected. The synthesis protocol included Fmoc deprotection with 20% piperidine in dimethylformamide (1 + 10 min) and couplings with three equivalents each of Fmoc-amino acid, di-isopropylcarbodi-imide and 1-hydroxybenzotriazole in dimethylformamide for 45 min. After coupling pGlu, the peptide was fully deprotected and cleaved from the resin by treatment with trifluoroacetic acid/water (9 : 1, v/v; 30 min; 4 °C) followed by precipitation with chilled diethyl ether. The crude material was purified by preparative HPLC on a Vydac C_8 column (250 × 20 mm; 10 μm) using a 10–20% linear gradient of B into A (A, 10 mM ammonium acetate, pH 6.5; B, 80% acetonitrile in 10 mM ammonium acetate, pH 6.5), at 25 mL·min^{-1}. Fractions judged to be homogeneous by analytical HPLC were pooled and lyophilized to yield the target peptide (global yield 5%, HPLC purity 90%), with correct amino-acid analysis and MALDI-TOF mass spectrum [*m*/*z* calculated for (M-H$^+$)$^-$, 1315.4 Da; found, 1315.2 Da]. The peptides EQFDDY(SO_3H)GHMRFamide [perisulfakinin (Pea-SK)] and proctolin (RYLPT) were from Bachem AG (Bubendorf, Switzerland), and human CCK-8 [DY(SO_3H)MGWMDFamide] was from Neosystem (Strasbourg, France). Nonsulfated Pea-SK and Lem-SK-II were synthesized using conventional Fmoc chemistry. Deprotection and coupling steps were as for Lem-SK-II. After cleavage, the identity and purity (≈ 90%) of each peptide were assessed by amino-acid analysis, MALDI-TOF MS, and HPLC. These data were used to quantify this and other synthetic peptides for the bioassays.

Myotropic assay

Peptides were tested on hindgut and foregut of *B. germanica* females prepared in a standard organ bath [9]. The composition of the bath was: 154 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 22 mM glucose and 5 mM Hepes, pH 6.8. An FSG-01 transducer (Experimetria Ltd, Budapest, Hungary) was used for isometric recording. The activity was calculated as the difference of the mean of the force produced by the tissue in the minute after and before the treatment. As a reference, an assay with proctolin (1 nM) was carried out on each preparation.

Feeding assay on goldfish

Experiments to study the effects of Pea-SK and CCK-8 on the feeding behaviour of goldfish (*Carassius auratus*) were carried out as previously reported [10]. Briefly, after starvation for 48 h, male and female goldfish (20–30 g) were intraperitoneally injected with saline, human CCK-8, or Pea-SK in saline. They were then fed *ad libitum* for 30 min, and the number of previously weighed individual pellets of food (commercially prepared fish pellets) consumed by each animal was recorded.

Sequence alignments and cluster analysis

The sequence of the SK isolated from *B. germanica* was aligned with those of other SKs identified in insects [11] and in the crustacean *Penaeus monodon* [12]. We also added the sequences of a selection of regulatory peptides with a similar C-terminal sequence including a biologically critical sulfated tyrosine for comparison. This selection comprised vertebrate gastrins and CCKs (the shortest forms in every case), caeruleins from amphibian skin secretions (from *Hyla caerulea* and *Xenopus laevis*), and cionin from the tunicate *Ciona intestinalis* [13]. In addition, we included in the alignment the five lymnaDFamides identified [14] in the snail *Lymnaea stagnalis*, and a selection of invertebrate FMRFamides [15]. A BLAST search in protein databases [16] using the above compounds did not find related sequences of other families of peptides. Software from the Genetics Computer Group (GCG, version 9.1, University of Wisconsin [17]) was used for sequence analysis. Sequence alignment was carried out with PILEUP, allowing a value of 4 for the gap creation penalty, which aligns the sulfated Tyr of vertebrate and invertebrate peptides. The cluster analysis was carried out with the Phylogeny Inference Package (PHYLIP, version 3.57c) [18], using amino-acid sequences to construct an unrooted UPGMA tree based on a Dayhoff PAM matrix [19].

RESULTS

Isolation of a factor that inhibited food intake

Preliminary assays of food-intake inhibition were carried out using the 17–40% acetonitrile fractions from the solid-phase extraction of brains from 5- to 7-day-old females. The weight of carrot ingested in controls was 1906.8 ± 194.9 μg compared with 1118.9 ± 234.4 μg in specimens treated with 10 brain-equivalents ($n = 13$), which shows that food intake was significantly inhibited by 41.3%.

To isolate the factor responsible for this biological activity, the crude brain extract from 5- to 7-day-old females was processed through four consecutive HPLC steps until a homogeneous peak reflecting food-intake inhibition was isolated (Fig. 1). In the first three steps, biological activity was measured with the carrot test in every 2-min fraction, and the active fractions (those with statistically significant inhibition) were collected and used for the next chromatographic step. The number of brain equivalents used in the carrot tests, their results, and the percentage of acetonitrile corresponding to the active fractions are summarized in Fig. 1. In the fourth step, carrot tests were carried out on isolated peaks. Only the peak corresponding to 19.6% acetonitrile gave an apparent 10% inhibition, which was not statistically significant (ingested carrot in controls and treated specimens 1608.4 ± 226.8 μg and 1450.7 ± 198.8 μg, respectively; $n = 10$; *t* test, $P = 0.6$). As none of the other peaks or areas between peaks gave any

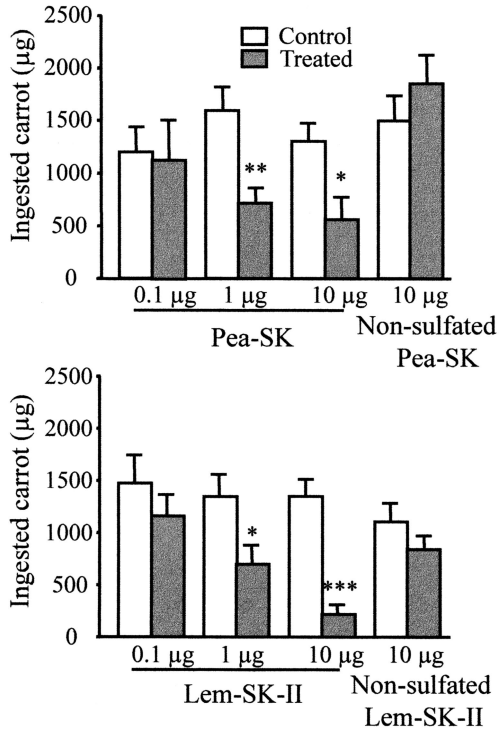

Fig. 2. Results of the carrot assay of food-intake inhibition in *B. germanica* for the peptides Pea-SK and Lem-SK-II, and their respective nonsulfated analogues. Results are expressed as mean ± SEM ($n = 12–14$). Asterisks indicate significant differences (Student's *t*-test): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

inhibitory activity, the peak at 19.6% acetonitrile was selected for identification.

Identification of the active compound

The peak at 19.6% acetonitrile from the fourth HPLC step was analysed by MS showing a $(M\text{-}H)^-$ mass (negative mode) of 1521.33. When analysed in the positive $(M\text{-}H)^+$ mode, a mass of 1443.69 was obtained. This difference of 80 units suggests that a sulfate group was lost in the positive mode. Amino-acid analysis by Edman degradation gave the sequence EQFDDYGHMR, which, on comparison with the molecular mass, indicates the presence of a final indeterminate residue. Taking together the sequence from the Edman degradation and MS data in both positive and negative modes, the only possible sequence is EQFDDY(SO_3H)GHMRFamide. The amidation at the C-terminus is justified by the accuracy of MS. The identification was further confirmed by coelution of the synthetic peptide with the native peptide in all HPLC steps.

This sequence corresponds to a peptide belonging to the SK family, identical with Pea-SK, which had been isolated from the corpora cardiaca of the cockroach *Periplaneta americana*, where it showed myotropic activity on the hindgut [20]. Given that a second SK [pQSDDY(SO_3H) GHMRFamide, leucosulfakinin-II (Lem-SK-II)], had been described in *P. americana* [20] and the cockroach *Leucophaea maderae* [21,22], it was also expected in *B. germanica*. However, we failed to detect Lem-SK-II, in sulfated or nonsulfated form or with the methionine oxidized, although we used the respective synthetic compounds as a reference.

Activity of synthetic peptides as food-intake inhibitors

A synthetic version of the peptide Pea-SK was tested with the carrot assay on *B. germanica* at concentrations of 0.1, 1 and 10 µg per specimen. Doses of 10 and 1 µg inhibited food intake by ≈60%, whereas the 0.1 µg dose was also inhibitory, but not significantly (Fig. 2). The nonsulfated analogue of Pea-SK was inactive at 10 µg (Fig. 2). Responses of Lem-SK-II were similar, although 10 and 1 µg doses resulted in significant inhibition of 84% and 48%, respectively (Fig. 2).

Effect of synthetic peptides on gut motility

As SKs have been described in other cockroaches as myotropic peptides acting on gut tissues, we studied the effects of Pea-SK and Lem-SK-II on foregut and hindgut motility in *B. germanica*. Both showed myostimulatory activity on foregut and hindgut preparations (Fig. 3). In all cases, the activity was comparable to that of proctolin (Fig. 3), which was used as a reference. The activity of nonsulfated Pea-SK and Lem-SK-II was at least three orders of magnitude lower than that of sulfated peptides (data not shown).

Activity of proctolin as a food-intake inhibitor

The myotropic activity of SKs in *B. germanica* may suggest that the inhibition of food intake induced by these peptides may be a simple consequence of their activity on gut motility. This led us to test a typically myostimulatory peptide, proctolin, in the carrot assay. However, proctolin did not show any antifeedant property when tested at 10 µg

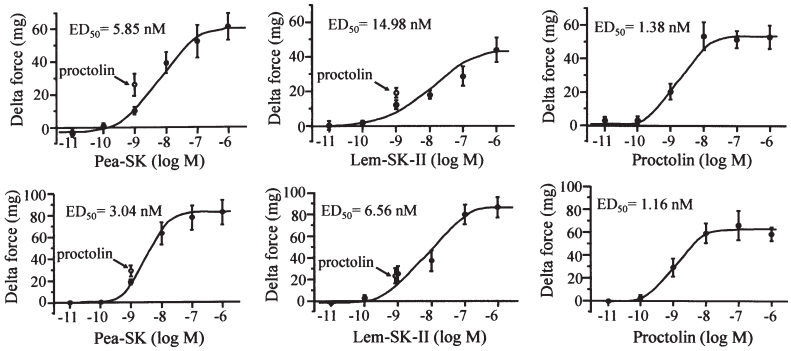

Fig. 3. Effect of Pea-SK, Lem-SK-II and proctolin on myotropic assay using *B. germanica* female foregut (top) and hindgut (bottom). As an internal control, an assay using 1 nM proctolin was performed for each SK preparation. Results are expressed as mean ± SEM ($n = 5–7$).

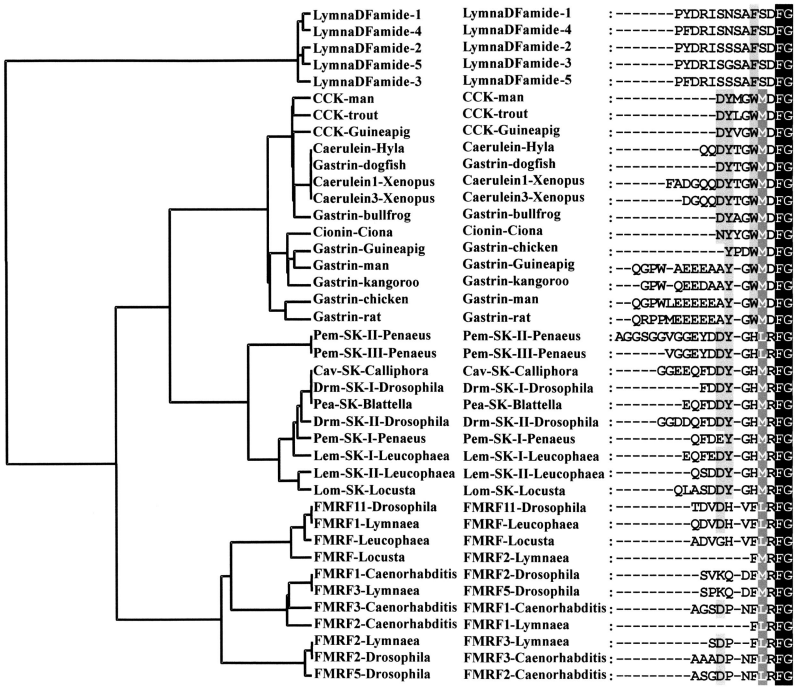

Fig. 4. Alignment and cluster analysis. Right side: alignment of the amino-acid sequences of lymnaDFamides (DF), cholecystokinins (CCK), caeruleins, gastrins, cionin, sulfakinins (SK) and FMRFamides (FMRF) generated by the PILEUP program from the GCG package, allowing a value of 4 for the gap creation penalty. Amidation at the C-terminus is indicated with the amino acid G, following the cDNA translation criterion; with the same criterion, pQ (which is the first residue in Pem-SK-I, Lem-SK-II and Lom-SK), is indicated as Q. In mammalian CCKs and gastrins, the shortest forms (8 and 17/16 residues, respectively) were used. For nonmammalian gastrins, we used the shortest native sequences. Reptilian gastrins were not included because they are completely nonsulfated [13]. Most of the SKs and gastrin–CCK sequences also occur in other closely related species (for example, Pea-SK which occurs in *B. germanica* and *P. americana*). Left side: cluster analysis from the alignment. The UPGMA tree was constructed from a Dayhoff PAM matrix.

(ingested carrot in controls and treated specimens 1946.1 ± 266.2 and 2533.1 ± 348.9 μg, respectively; $n = 12$ in both cases) or 25 μg (ingested carrot in controls and treated specimens 1474.5 ± 305.2 and 1747.9 ± 358.0 μg, respectively; $n = 24$ in both cases).

Antifeedant activity of CCK in German cockroach and Pea-SK on goldfish

Given the similarity of the sequences in SKs and gastrin–CCKs, the activity of a representative peptide of the vertebrate gastrin–CCK family was tested as a feeding inhibitor in *B. germanica*, and insect Pea-SK was tested in a vertebrate, using a feeding assay on goldfish. Human CCK-8, at a dose of 10 μg, did not inhibit feeding in *B. germanica* (ingested carrot in controls and human CCK-8-treated specimens 1715.7 ± 276.4 and 2063.5 ± 324.5 μg, respectively; $n = 11$ in both cases). Similarly, Pea-SK tested at a dose of 5 $\mu g \cdot g^{-1}$ did not inhibit feeding in goldfish (ingested food pellets in controls and Pea-SK-treated specimens 12.78 ± 0.44 and 14.99 ± 1.53 $mg \cdot g^{-1}$, respectively; $n = 6$ in both cases). As a positive control of the goldfish feeding assay, we observed that human CCK-8, tested at a dose of 0.5 $\mu g \cdot g^{-1}$, significantly inhibited food intake by 49% (ingested food pellets in controls and human CCK-8-treated specimens: 13.59 ± 0.93 and 6.92 ± 1.28 $mg \cdot g^{-1}$, respectively; $n = 9$ in both cases; Student's *t*-test, $P = 0.002$).

Comparison of insect SK with vertebrate CCK

The structure of SKs, with a typical C-terminal sequence DY(SO_3H)GHMRFamide, and its antifeedant activity reported here, suggested a possible evolutionary association with vertebrate CCKs, which have a similar typical C-terminal sequence DY(SO_3H)MGWMDFamide and are satiety promoters. Therefore, we compared the available arthropod SKs with a selection of vertebrate CCKs and related vertebrate peptides, such as gastrins, caeruleins and cionin [13]. We also included lymnaDFamides, and a selection of arthropod FMRFamides in the comparison. LymnaDFamides are tridecapeptides that were detected with an antiserum that recognized the biologically active C-termini of gastrin and CCK [14], and they share with the latter peptide family the two C-terminal residues. FMRFamides share with SKs and gastrins-CCKs the C-termini L/MXFamide [15].

Alignment of these peptides (Fig. 4) reveals that lymnaDFamides are clearly separated from the three remaining groups, one formed by gastrins plus CCKs, caeruleins and cionin, another formed by SKs, and the last one by FMRFamides. Of these three groups, the one most closely related to SKs is that containing gastrins plus CCKs plus caeruleins plus cionin, given that, in terms of consensus, they share the positions 13 (D), 14 (Y), 16 (G), 18 (M), 20 (F) and 21 (G). In spite of the scarce number of positions available, we carried out a cluster analysis based on the Dayhoff matrix, which gave an UPGMA tree (Fig. 4) which reflects the relationships suggested by the alignment.

DISCUSSION

The search for factors that regulate the feeding cycle in the adult female *B. germanica* led to the identification of the peptide EQFDDY(SO_3H)GHMRFamide (Pea-SK). It was isolated from *B. germanica* brain extracts by monitoring the antifeedant activity of fractions from HPLC separation. Antifeedant activity was measured by a food-intake assay based on colorimetric measurement of carotenoids from ingested carrot.

The peptide is identical to Pea-SK identified in the cockroach *P. americana* as a myotropic factor [20]. In

P. americana, as well as in the cockroach *L. maderae* [21,22], another SK with the sequence pQSDDY(SO_3H)GHMRFamide (Lem-SK-II) has been identified, suggesting that this peptide is also present in *B. germanica*. However, we failed to isolate it from brain extracts. Additional SKs have been reported in other insects, such as the orthopteran *Locusta migratoria* and the dipterans *Neobellieria bullata*, *Drosophila melanogaster*, *Calliphora vomitoria* and *Lucilia cuprina* [11]. New SKs have recently been isolated from the crustacean *P. monodon* [12]. All peptides identified to date have between 19 and 9 amino acids and share the C-terminal sequence D/ED/EY(SO_3H)GHM/LRFamide. The gene expressing the SK precursor was cloned and sequenced in *D. melanogaster* [23]; it was shown to encode three peptides flanked by prohormone-processing sites, two of which belong to the SK family. The SK genes of *C. vomitoria* and *L. cuprina* [11], which were later cloned and sequenced, are organized in a similar way.

SKs were originally detected in the cockroach *L. maderae* by hindgut motility measurements [21,22]. Myotropic properties were also reported in *L. migratoria*, and a new SK was described in this locust [24]. More recently, a number of SK forms with myotropic properties in the hindgut and heart tissues have been reported in *P. americana* [25]. However, in the dipteran *C. vomitoria*, SKs do not elicit myotropic effects in gut tissues [26]. This is explained by *in situ* hybridization and immunocytochemical studies, which show that only four pairs of neurons that express the SK gene are located in the brain, whereas their axons appear to be restricted to the central nervous system [11,26]. Similar restricted patterns of distribution, suggesting a neurotransmitter and/or neuromodulator function for SKs, have been described in immunocytochemical studies on other species, such as the fruitfly *D. melanogaster*, the moth *Manduca sexta*, and the cricket *Teleogryllus commodus* [27]. In addition, and only in *P. americana*, SK-like material has been localized in axons of the stomatogastric nervous system innervating the crop (suggesting a myomodulatory role) and in the cardiacocommissural organ, which forms the floor of the aorta (suggesting neurohemal release in this area) [27]. Other studies on this cockroach have revealed SK-immunoreactive material in endocrine cells of the midgut [28]. The only cytochemical study in *B. germanica* reported CCK-8 immunoreactivity in brain and the retrocerebral complex [29]. It is not clear whether the CCK-8-like material revealed corresponds to SK [14], although the neuronal pattern described is similar to that observed in *P. americana* using SK antiserum [27].

This study shows a clear effect of cockroach SKs as inhibitors of food intake. For the two SKs tested, Pea-SK and Lem-SK-II, the activity was dose-dependent, with 50% inhibition at doses of 1 μg, whereas the nonsulfated analogues were inactive. Effective doses of 1 μg *in vivo* can be considered low for an insect regulatory peptide, given their low stability in the haemolymph. Because of the sequence similarity of SKs and gastrin–CCKs, the SK pQLASDDY(SO_3H)GHMRFamide, identified from *L. migratoria* [24], was recently tested as a feeding inhibitor in *Schistocerca gregaria*, by weighing the food before and after the 20-min feeding test. Doses of 1.5 μg reduced food consumption by about 50% [30], supporting the antifeedant properties of these compounds in insects.

Given the myotropic activity of SKs in orthopteroids, we aimed to determine whether the antifeedant activity was simply a consequence of the stimulation of gut motility. To this end, we chose proctolin, a classical model of myotropic peptides in insects [15], and studied its response. Proctolin elicited the expected myotropic activity but did not show antifeedant effects. This suggests that SKs have genuine properties of food-intake inhibitors, in addition to being myotropic peptides.

The inhibitory effects of insect SKs on food intake raised the question of whether these peptides are homologous to vertebrate gastrin–CCKs, as suggested previously [20–24], or simply analogous [11–13,26]. From a biological point of view, CCKs are key regulators of satiety in vertebrates. Recent studies have demonstrated that the main target of CCK in feeding regulation is the vagus nerve, which conveys parasympathetic activity between the gut and the brain. Gastric vagal afferents linked to CCK terminate on cell bodies in the nucleus tractus solitarius, the primary area of the brain that processes the information from the gastrointestinal tract [1]. In insects, most studies have localized SKs in the brain, although in the cockroach *P. americana*, an antibody specific for Pea-SK revealed SK-immunoreactive material in endocrine cells of the midgut [28]. A further parallel is the stimulatory effect of SK on the release of the digestive enzyme α-amylase described in the beetle *Rhynchophorus ferrugineus* [31], which is reminiscent of the secretagogue action of CCK in mammals.

These biological parallels led us to carry out heterologous feeding assays by testing human CCK-8 on the German cockroach, and insect Pea-SK was assayed on a vertebrate model, the goldfish *C. auratus*. In both cases the results were negative, even at a very high dose of the corresponding peptide. Even though CCKs have a C-terminal sequence [DY(SO_3)XGWMDFamide] that is similar to that of arthropod SKs [D/EY(SO_3)GHM/LRFamide] and sulfation of Tyr is strictly necessary for biological activity in both cases, the negative results of heterologous assays are not surprising. Indeed, the WMDFamide tetrapeptide sequence of CCKs, which is biologically essential, cleverly differs from the equivalent HM/LRFamide of SKs. The change from Trp to His and Asp to Arg requires a minimum of two base substitutions, and the conformation and charge of the two tetrapeptides are considerably different [12]. However, analogues of CCK in which Asp has been replaced by the neutral amino acid Pro retain a modest but significant 10% of the potency of CCK in pancreatic binding and satiety assays in rats, and replacement of Arg by Pro in the equivalent position of the locust SK, pQLASDDY(SO_3H)GHMRFamide, also results in retention of a significant 10% of the myostimulatory potency in the insect hindgut assay [31].

Finally, and strictly from a structural point of view, the alignment and cluster analysis of a selection of related peptides (including invertebrate FMRFamides and lymnaDFamides) indicates that the gastrin–CCK family of peptides (which also includes cionin and caeruleins) is the closest relative of SKs. This suggests that SKs and gastrin–CCKs are homologous, and that mechanisms of feeding regulation involving these types of regulatory peptide may have been conserved throughout evolution between insects and vertebrates.

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